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Signed W. Russell

Dated 15 May 1995

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31 MAY 1994
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Your reference IMA/FP5130596

9410870.1

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Request for grant of a Patent

Form 1/77

Patents Act 1977

❶ Title of invention

1 Please give the title of the invention: MODIFICATION OF BOTULINUM TOXINS FOR USE AS TRANSPORT PROTEINS

❷ Applicant's details

❑ **First or only applicant**

2a If you are applying as a corporate body please give:

Corporate name: THE IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY AND MEDICINE

Country *(and State of incorporation, if appropriate)*: UNITED KINGDOM

2b If you are applying as an individual or one of a partnership please give in full:

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Address: THE IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY AND MEDICINE
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UK postcode *(if applicable)*: SW7 2AZ

Country: UNITED KINGDOM

ADP number *(if known)*: 04038452001

2d, 2e and 2f: *If there are further applicants please provide details on a separate sheet of paper.*

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Agent's name MEWBURN ELLIS

Agent's address YORK HOUSE
23 KINGSWAY
LONDON

Postcode WC2B 6HP

Agent's ADP number 109006

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4 Agent's or applicant's reference number *(if applicable)* IMA/FP5130596

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5 Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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Yes ☐ No ☒ ➡ ***go to 6***

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Continuation sheets for this Patents Form 1/77			
Claim(s)		Description	28
Abstract	1	Drawing(s)	4

8b Which of the following documents also accompanies the application?

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MODIFICATION OF BOTULINUM TOXINS FOR USE AS TRANSPORT PROTEINS

The present invention relates generally to the field of receptor-targeted biochemical delivery systems. More specifically, this invention relates to the use of modified botulinum toxins as vehicles for delivering chemical compounds to cells bearing toxin receptors.

Botulinum toxins (BoNT) are a family of potent neurotoxins that induce paralysis by mechanisms that involve the inhibition of neurotransmitter release. These *Clostridial* neurotoxins are initially produced as single-chain proteins of 150 kDa. Proteolytic cleavage then generates an active dichain molecule having a 100 kDa heavy (H) chain and a 50 kDa light (L) chain that are linked by a single interchain disulfide bond. Whereas the H chain mediates both the binding of the toxin to neuronal cell surface receptors and translocation of L chain into cells, the L chain is responsible for blocking the release of neurotransmitters. Although botulinum toxins cause persistent inhibition of synaptic vesicle exocytosis, they are not known to impair other aspects of neuronal cell physiology.

Seven serologically distinct isoforms of BoNT are produced by *Clostridium botulinum*. The seven botulinum toxin species are designated as BoNT/A-G. Botulinum type B neurotoxins are known to be zinc-dependent proteases. In *EMBO J.* 12:4821 (1993), Blasi et al. proposed that the botulinum neurotoxin serotypes have evolved distinct substrate specificities while retaining a common protease activity. Whereas BoNT/A-G may cleave different protein components of the exocytotic machinery, the consequence is the same. Hence, it is believed that the various botulinum toxin isoforms are both structurally and functionally related.

Tetanus toxin (TeTx) is another *Clostridial* neurotoxin that is related to the Botulinum toxins. Each of these toxins use different cell surface receptors and cleave a variety of protein targets that are involved in vesicle function. Tetanus toxin, BoNT/B and BoNT/D all cleave VAMP-2, or Synaptobrevin-2. The BoNT/A and BoNT/E toxins cleave the membrane-associated SNAP-25 protein, while BoNT/C cleaves syntaxin. Tetanus, BoNT/D and BoNT/F have been reported to cleave Cellubrevin. The BoNT/F

toxin cleaves VAMP, or Synaptobrevin. The target protein of the BoNT/G toxin is not yet identified.

As described above, all of the *Clostridial* neurotoxins bind to different cell surface receptors. However, all of the toxins bind to and affect cholinergic motor neurons. Other neurons can also be affected, particularly at high toxin concentrations. The similar functional properties of the *Clostridial* toxins reflect common structural features.

Certain zinc-dependent endoproteases contain the conserved amino acid sequence HExxH. In thermolysin, zinc binding is achieved via His^{142} and His^{146} within this motif, together with Glu^{166}; the fourth ligand is water. Comparison of the sequence of L chain with those of thermolysin and other zinc endoproteases has revealed the presence of the same consensus motif.

The role of Glu^{234} within this motif in the L chain of TeTx has been studied using site-directed mutagenesis and an *in vitro* assay for the proteolysis of cellubrevin. In *Nature* **364**:346 (1993), McMahon et al. demonstrated that cellubrevin was not cleaved when COS cells were cotransfected with mutant L chain (Glu^{234} substituted by Gln) and cellubrevin DNA constructs.

The present invention exploits the target specificity of the botulinum neurotoxins as a means for specifically delivering chemical agents to neuronal cells. Whereas the enzymatic properties of these toxins could be a disadvantage in such applications, the following invention overcomes this limitation.

One aspect of the present invention relates to a chemical conjugate for treating a nerve cell related disorder. This conjugate includes an active or inactive botulinum toxin having specificity for a target nerve cell. The toxin is conjugated to a drug or other bioactive molecule without affecting the toxin's ability to enter the target nerve cell.

Further aspects of the present invention will be apparent to one having ordinary skill in the art upon reference to the ensuing detailed description.

Figure 1 is a schematic representation of the recombinant light chain expression construct, pCAL. This was produced by insertion of the L chain gene between the *Bam* HI and *Sal*I restriction sites at the polylinker of the vector pMAL-C2. The vector

contains the inducible P_{tac} promoter positioned to transcribe the *malE-LacZα* gene fusion. The *lacI*^{q} gene encodes the lac repressor which turns off trascription from P_{tac} until induction by IPTG. The *rrnB* terminator prevents transcription from interfering with plasmid replication. Amp^r encodes β-lactamase for ampicillin resistance. M13-ori and pBR322ori indicate the origins of DNA replication. The Factor X_a cleavage site and L chain start are denoted by arrows.

Figures 2A and 2B are line graphs illustrating that recombinant L chain or its fusion protein inhibit catacholamine release from permeabilized chromaffin cells. Figure 2A is before Factor Xa cleavage and Figure 2B is after Factor Xa cleavage. Cells were permeabilised by incubation for 15 minutes with 20 μM digitonin in KGEP buffer (139 mM K^+ glutamate, 5 mM ethylene glycol-bis[β-aminoethyl ether] N,N,N',N'-tetraacetic acid [EGTA], 2 mM ATP, 2 mM $MgCl_2$, 20 mM piperazine-N,N'-bis-[2-ethanesulfonic acid] [PIPES] pH 6.5) containing the indicated concentration of native BoNT A (o) or (Δ) or recombinant L chain fusion protein before (•). or after (▲) cleavage with Factor Xa. Following a quick rinse with KGEP, cells were incubated for 15 minutes with KGEP with or without 20 μM free Ca^+. An aliquot was then removed form each well and assayed for catecholamine content by a fluorometric method. Catacholamine remaining inside cells was calculated after Tx-100 solubilisation, and secretion was calculated as a percentage of the total cell content (=remaining + released). Catecholamine in the Ca^{2+} -free buffer was subtracted from that secreted into that containing 20 μM Ca^{2+} to calculate evoked release.

Figure 3 is a line graph showing the effect of native and recombinant L chain on nerve-evoked neuromuscular transmission at motor end plates once reconstituted with the native H chain of BoNT A. When applied to mouse phrenic nerve-hemidiaphrams, BoNT A chain reconstituted with recombinant L chain, not previously purified from MBP after Factor Xa cleavage (1.6 nM; O) blocked neuromuscular transmission with approximately the same efficacy as the native resonstituted L and H chains (2.0 nM; ∇). The concentrations of the reconstituted material was calculated following the quantification of the amount of the 150 kDa di-chain material present by SDS-PAGE and densiometric scanning. The tissues were bathed in Krebs-Ringer medium aerated with 95% O_2 and 5% CO_2 maintained at 24°C. All points shown are the average of at least three separate experiments ± SD.

Figure 4 is a schematic representation of the chemical synthetic scheme used to link the drug Vesamicol to a TeTx transporter using Maleimide.

The present invention relates to the use of modified botulinum neurotoxins as transporters for the delivery of linked pharmacologic compounds. Among the compounds that will be linked to the toxin transporters are visualizable agents bearing fluorochromes, and drugs of therapeutic value. The contemplated cell populations that will be targeted by the toxin transporters include those which express cognate toxin receptors.

We discovered that an effective drug delivery agent may be prepared by the mutagenesis of a single amino acid position in the L chain of a botulinum neurotoxin to inactivate its protease activity and then attaching a drug to that inactivated neurotoxin. Despite this abolition of enzymatic activity, the mutagenized toxin advantageously retained the ability to bind its cognate cell surface receptor. In addition, we have discovered other unexpected properties of the attenuated Botulinum toxin molecule.

Significantly, we have discovered that both the heavy and L chains of the botulinum neurotoxins are required for optimal receptor-ligand interaction. In light of this finding, we reasoned that a toxin transporter would advantageously comprise both chains of the dichain molecule. Since the toxic properties associated with the L chain molecule could interfere with the therapeutic effect of a drug that was covalently linked to the toxin transporter, we endeavored to create an attenuated L chain molecule that did not inhibit binding of the dichain molecule to the cognate toxin receptor. We discovered this could be accomplished, with apparently minimal disruption to the folded structure of the L chain protein, by mutation of a single amino acid position.

Accordingly, use of the inactivated botulinum toxin molecule as a vehicle that can be covalently linked to a drug has been explored. Reconstituted toxin, having an inactivated L chain disulfide-bonded to a native H chain retained the ability to specifically interact with target receptors. Hence, the inactivated and chemically modified toxin complex can be used as a system for delivering linked chemical compounds to neuronal cells that express cell surface receptors for the toxins. Experiments wherein reconstituted native botulinum heavy chain and non-attenuated recombinant light chain were injected into mice demonstrated that the molecule could

act locally to induce flaccid paralysis. This result indicates that molecules reconstituted by the methods disclosed herein still retain their native ability to be internalized and effect neuronal cell functions.

As detailed below, an inactive botulinum L chain can be reassociated with the native H chain to form an inactive dichain toxin. This dichain molecule can serve as a receptor-targeted carrier for various chemical compounds. Due to the specificity of the H chain for its target receptor, a drug compound can be efficiently and specifically delivered to neuronal cells.

In the development of the present invention, the gene encoding Botulinum chain was modified at the 5' end by the addition of maltose protein binding domain sequence. This domain, therefore, was added to the N-terminal portion of the botulinum protein. Following expression in *E. coli*, the recombinant fusion protein (called MBP-L chain) was purified by affinity chromatography. Proteolysis allowed separation of the L chain and the MBP domains. The purified L chain was then associated with purified H chain that had been isolated from *C. botulinum*-derived botulinum toxin to generate a dichain. This reconstituted botulinum molecule displayed activity characteristic of the native toxin. Parallel findings have also been made using the recombinant tetanus protein after reassociation with native H chain protein.

As disclosed herein, separate BoNT/A L chain molecules were modified at either the HIS^{227} or Glu^{224} residues. These modifications in the BoNT/A L chain caused the loss of proteolytic activity against cellular target substrates. Of course, many sites in the botulinum toxin zinc binding motif might be modified to provide the results discussed herein. The scope of the present invention is meant to encompass any attenuated botulinum toxin that is used as a transporter molecule.

By the methods described herein, mutant botulinum toxins can be synthesized. These toxins will retain their ability to bind neurons, even in the absence of an associated protease activity. These attenuated toxins can therefore facilitate the production of novel systems for the specific delivery of chemical agents to target neurons.

The mutagenized and enzymatically inactive dichain botulinum toxins described herein will advantageously serve as neuropharmacologic transport agents for transporting chemical compounds to neuronal cells that express cell surface receptors for the toxins. Bonding of chemical agents to the transporter protein is requisite for

proper implementation of the invention. Such chemical agents can be pharmacological agents, chemotherapeutic agents or visualizable agents that can be detected by light or other form of electromagnetic radiation.

The localized activity of the botulinum toxins can be exploited in therapeutic protocols employing modified toxin-transporters. In particular, modified toxins based on one of the botulinum serotypes are expected to remain localized at the site of injection. The inactive botulinum toxin transporter will be primarily used to deliver drugs that target the peripheral motor nerve terminal. Therefore, diseases which affect limited muscle groups will be most appropriately treated using the BoNT/A based transporter. Transporters based on other botulinum toxin serotypes may also be effective for this purpose.

Diseases that may benefit from such therapies include, but are not limited to, tardive dyskinesia, spastic colitis, essential tremor, gastric smooth muscles, achalasia (abnormal contractions of the esophagus), localized spasticity, painful muscle spasms localized to back or other muscle groups, temporal mandibular disorders, spasmodic dysphonia (overactive vocal chords), swallowing disorders, tension headaches, spasmodic torticollis, post stroke or traumatic brain injury induced spasticity, dystonias of large muscle groups, cardiovascular smooth muscle (i.e., arteriole), and sphincter smooth muscle found in various organs (gall bladder, urinary bladder, rectum, etc.).

Table 1 outlines potential therapeutics related to the present invention. The entries in this table describe specific drug classes that will be linked to the botulinum toxin molecules. Although we have illustrated the value of using inactive dichain botulinum toxin type A proteins as transporters, the toxins of all other botulinum serotypes (B-G) could similarly be used. The different serotypes of botulinum toxin utilize different presynaptic receptors. Hence, we foresee the use of different toxin serotypes as transporters that can advantageously be used to provide specificity for drug delivery. This would be particularly useful if some tissues selectively expressed one receptor more than any other. Alternatively, two transporters could be used to deliver different therapeutic agents to the same target area of the body. This latter approach would advantageously reduce competition between different toxin ligands at the receptor site.

Additionally, the use of both native and recombinant wild type botulinum neurotoxin proteins is intended to fall within the scope of the

claims of our invention. In such applications, the enzymatic activity possessed by the L chain portion of the drug transporter will provide an added therapeutic advantage by virtue of its neurotoxic properties. For example, a drug that blocked nerve function could be linked to a wildtype botulinum toxin molecule to provide a compound that had a double action. The botulinum toxin molecule would provided its wildtype neuronal inhibitory effect, while the drug acted at its target site in the cell.

Examples of neuromuscular maladies that will be investigated as therapeutic targets using active neurotoxins linked to drug molecules include: focal dystonias, spasticities due to stroke or traumatic brain or spinal cord injury, blepharospasm, strabismus, cerebral palsy and back pain due to muscle spasms.

As indicated below, some of the drugs selected act intracellularly while other act extracellularly. As discussed herein, the intracellular drugs can be bound to a botulinum toxin carrier and efficiently internalized. However, drugs with extracellular actions can also be used in the present invention. We have discovered that reduced, alkylated botulinum toxin molecules can bind to the exterior of the cell, but will not be internalized (de Paiva et al., *J. Biol. Chem.* **268**: 20838-20844 (1993)). Thus, these reduced, alkylated molecules can be linked to extracellular-acting drugs and carried to the target cell surface. Once bound to the cell surface, enzymes such as esterases can cleave the drug from the toxin carrier thus releasing the drug in close proximity to the target cell.

A brief description of the various uses of the transporter forms coupled with representative drug classes are summarized below in Table 1.

Table 1

Therapeutic Uses of *Clostridial* Toxin Transporters

Transporter Molecule	Tissue Target	Drug Type	Mechanism of Action	Possible Clinical Outcome
Inactive-Intact-Botulinum Toxin	Peripheral muscle targeted by injection	Ribozyme or oligonucl-eotide	Prevent synthesis of critical nerve component needed for the neural transmitter exocytosis or nerve regrowth to reform the synapse at the neuromuscular junction. (i.e., block nerve muscle communication to establish stable synapse). Alternatively, block synthesis of ion channels. Another target is Choline acetyltransferase.	Prolonged skeletal muscle weakness or flaccidity, reduction of spasticity and/or pain. Duration: > 3 months
Inactive-Intact-Botulinum Toxin	Peripheral muscle targeted by injection	Nicotinic antagonist	Can be made to prevent molecule internalization, the drug could be attached which is released by the local acetylcholine esterase. this drug would then block the AChR on the muscle, released from the depot in the synapse.	Prolonged skeletal muscle weakness, reduction of spasticity and/or pain. Duration: hours to several days

Transporter Molecule	Tissue Target	Drug Type	Mechanism of Action	Possible Clinical Outcome
Inactive-Intact-Botulinum Toxin	Peripheral muscle targeted by injection	Neuronal calcium channel blocker	Block calcium entry into neuron and thus prevent release of transmitter.	Prolonged skeletal muscle weakness, reduction of spasticity and/or pain. Duration: hours to several days
Inactive-Intact-Botulinum Toxin	Peripheral muscle targeted by injection	Vesamicol or analog	Block transport of acetylcholine into the vesicle in the nerve terminal.	Prolonged skeletal muscle weakness, reduction of spasticity and/or pain. Duration: hours to several days
Active-Intact-Botulinum Toxin	Peripheral muscle targeted by injection	Ribozyme or oligonucleotide	Same target mRNA target as inactive transporter, above.	Prolonged skeletal muscle weakness of flaccidity, reduction of spasticity and/or pain. Duration: > 3 months
Inactive-Intact-Botulinum Toxin	Peripheral muscle targeted by injection	Acetylcholine esterase inhibitors	If a modification can be made to prevent molecule internalization, the drug released locally by hydrolysis and block acetylcholine esterase.	Enhanced muscle contraction. Could counter the effect of Botox.
Inactive-Intact-Botulinum Toxin	Peripheral muscle targeted by injection	Captopril and other zinc dependent protease inhibitors	Block proteolytic action of L chain.	Antagonize the effect of a Botox injection, if administered early enough.

The methods used to covalently couple the inactivated botulinum toxins and the chemical agents rely on conventional techniques that are familiar to those having ordinary skill in the art. The provision must be met however, that the domain of the compound that corresponds to the inactivated toxin retains the ability to specifically interact with cognate *Clostridial* toxin receptors on target cells.

Purified botulinum toxin type A has been clinically used as a neurotoxic agent. This compound, which is sold under the trade name BOTOX®, is manufactured by Allergan, Inc. (Irvine, California). A similar compound, Dysport is manufactured by Porton international. These agents are therapeutically used to produce localized chemical denervation/muscle paralysis. When chemically denervated in this fashion, the affected muscle atrophies and may develop extrajunctional acetylcholine receptors. It is believed that the affected nerve cells can sprout and reinnervate muscle tissue, thus rendering the paralytic activity of BOTOX® reversible.

Modified botulinum toxins, produced according to the methods described above, will be stored in lyophilized form in containers under vacuum pressure. Prior to lyophilization, the modified toxins will be combined with pharmaceutically acceptable excipients, including albumins and other appropriate agents as would be appreciated by those of ordinary skill in the art. Further information regarding such pharmaceutical preparations can be found in the "Physicians Desk Reference," published annually by Medical Economics Data of Oradell, New Jersey. The lyophilized material will be reconstituted with sterile non-preserved saline prior to intramuscular injection. This dissolved material will then be useful in the treatment of a variety of neuromuscular disorders as described above.

Methods of Linking Chemical Compounds to Light Chain Proteins

Whereas we contemplate that many different chemical compounds will be usefully bonded to toxin transporter molecules, a subset of these compounds will be neuropharmacologic agents or drugs. The following description therefore emphasizes methods of joining transporter proteins and drugs. However, those of ordinary skill in the art will appreciate the more generic term, "chemical compound" can reasonably be substituted for the term, "drug."

Many approaches are known for linking chemical compounds to the amino acid chains of proteins. We will use a linker molecule to separate the drug from the L chain peptide. As discussed above, we discovered that 7 amino acids can be attached to the N-terminus of the Botulinum neurotoxin L chain with out substantially affecting its functionality. For this reason, we will use the N-terminal portion of the botulinum toxin L chain as the compound attachment point.

It is known that most drugs have positions that are not sensitive to steric hindrance. In addition, the linkage process should not introduce chirality into the drug molecule. Further, the linker and the drug should be attached through a covalent bond. The distance between the L chain and drug can be adjusted by the insertion of spacer moieties. Preferable spacers have functional groups capable of binding to the linker, drug and L chain and serving to conjugate them.

Preferred Spacers:

1) $HOOC-(CH_2)_n-COOH$, where $n=1-12$, suitable for insertion at the amino terminal end of a peptide, to connect it with a linker on a drug molecule.

2) $HO-(CH_2)_n-COOH$, where $n>10$, suitable for attachment at the amino terminal of a peptide to connect the L chain with a linker on a Drug molecule.

3) $(C_6H_6)_n$, where $n>2$, suitable for attachment to join the L chain with a linker on the Drug molecule. The benzene rings provide a rigid spacer between the Drug and L chain. Of course, appropriate functional groups, for example as identified by X below, will be present on the benzene rings to link the drug and L chain.

Two different linker types are envisioned. In the first type, the Drug-Linker-L chain molecule remains intact after introduction into cells. In the second type, the Drug-Linker-L chain molecule is metabolized to free the drug after introduction into cells.

Linkers that remain intact after introduction

In one method, a cysteine residue is attached to the end of the L chain molecule by methods well known in the art. For instance, the gene construct that carries the L chain molecule can be mutated to include a cysteine reside at the N-terminal portion of the protein. A maleimide linker is then attached to the Cysteine residue by well known means.

In a second method, the linker is attached directly to the drug. A Drug-X moiety can have the following groups wherein X is OH, SH, NH_2, CONH, $CONH_2$. Of course,

the proper group would not be in an active site or sterically hindered. The following reaction would link the Drug-X to the linker molecule.

$$\text{Br-CH}_2\text{-Linker} \xrightarrow{\text{Drug-X}} \text{Drug-X-CH}_2\text{-Linker}$$

Once the Drug has a linker attached, the following reaction can be used to link the Drug to the Toxin. In this reaction, the toxin has an accessible Lysine group that is used as the attachment point for the Drug. As discussed hereinabove, an extra amino acid, such as lysine, can be readily added to the N-terminal portion of the L chain gene and used as the attachment point for a drug. In the following reaction, sodium cyanoborohydride is used to attach the linker to the lysine group on the L chain molecule.

$$\text{Drug-Linker-CHO} \xrightarrow[\text{Toxin-Lys}]{\text{NaCNBH}_3} \text{Drug-Linker-CH}_2\text{-NH-Toxin}$$

Drugs that are envisioned to work in the present invention are those that have a free -XH group and can act as neuroinhibitors. These neuroinhibitors can interfere with the over-production of neurotransmitters in some medical indications such that the nerves will be inhibited from firing. Appropriate drugs with -XH groups are aconitine, adenosine agonists/antagonists, adrenergics, anatoxin A, antiepileptics, baclofen, brachiotoxin, brefeldin A, brevetoxin, captopril, curare, dantrolene, doxorubin, diazepam, grayanotoxin, lidocaine, methocarbamol, methyllycaconitine, neosaxitoxin, physostigmine, psychosine, THA, tetrodotoxin, vesamicol and vigabatum.

Linkers that cleave after introduction

Depending on the Drug's mode of action, it may be important for the Drug to be released from the L chain after introduction. In this method, the Drug has a free -XH group that is the active site for synthesis with a linker. The -XH group could be an alcohol, phenol, amine, carboxylic acid or thiol group.

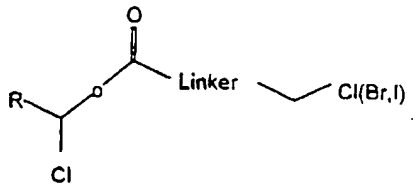
The general formula for linking a Drug to a toxin so that it will be metabolized after introduction is as follows:

$$\text{DRUG-XH} + \text{Linker} \xrightarrow{\text{Maleimide}} \text{DRUG-X-LINKER-Maleimide} \xrightarrow{\text{Toxin-SH}} \text{Drug-X-Linker-Maleimide-Toxin}$$

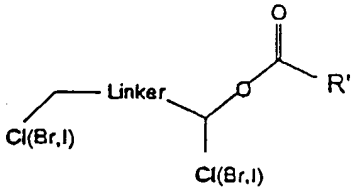
Where X can be OH, NH/NH_2, CO_2H, SH, $CONH_2$

Where the Linker can be A) or B) as detailed below:

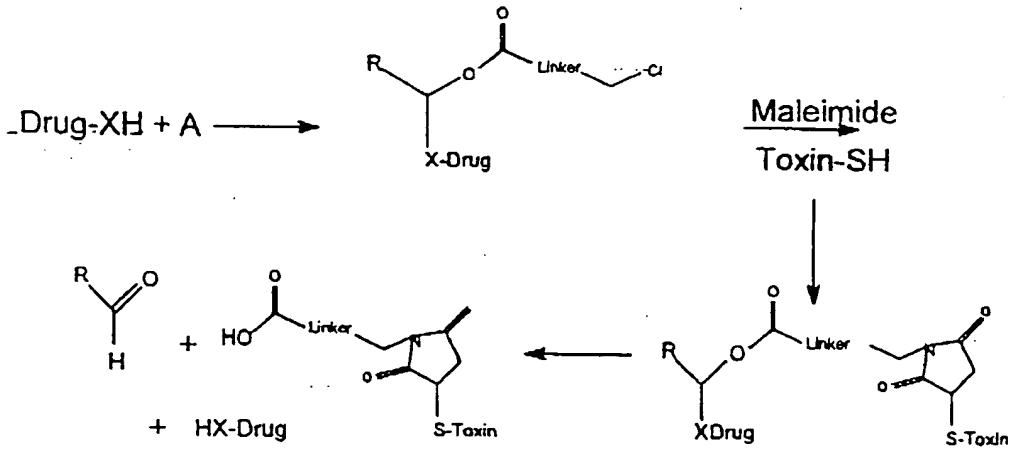
A)

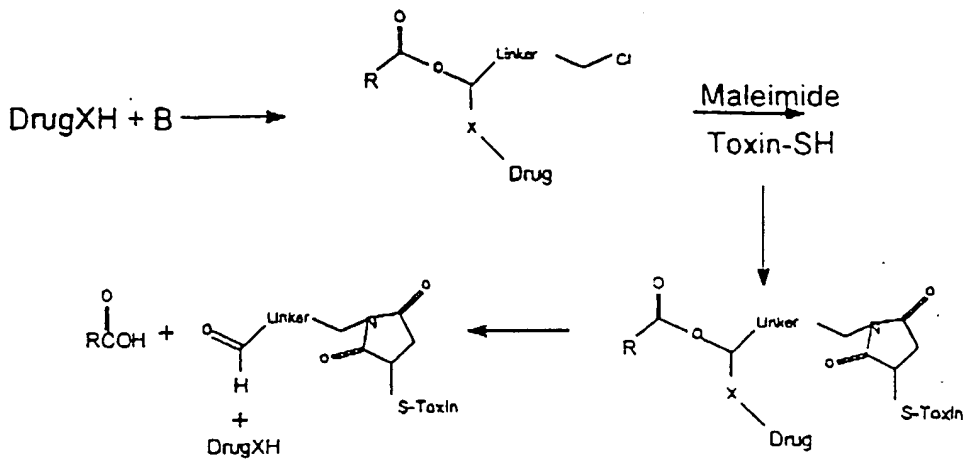


B)



The specific reactions with Linkers A or B are shown below.





Our strategy for linking ribozymes to the toxin transporters employs the free amine functional groups on adenosine and guanosine bases for linker attachment. In particular, our approach will be to incorporate modified adenosine or guanosine residues that are modified at their free amine positions with a linker that is in turn bound to the nitrogen position of succinimide. The structures of these modified nucleosides can be diagrammed as:

Sugar-Base-NH-Linker-Succinimide

Ribozymes are conventionally prepared by sequentially linking nucleosides in a defined order. The linking reaction occurs between the sugar moieties of the individual chemical units. Incorporation of a modified nucleoside, as described above, at either the 3' or 5' end of the ribozyme will provide a means for covalently linking to the toxin transporter according to the mechanism described previously.

Although other materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. General references for methods that can be used to perform the various PCR and cloning procedures described herein can be found in *Molecular Cloning: A Laboratory Manual* (Sambrook et al. eds. Cold Spring Harbor Lab Publ. 1989) and *Current Protocols in Molecular Biology* (Ausubel et al. eds., Greene Publishing Associates and Wiley-Interscience 1987).

The initial step in creating an inactivated botulinum toxin composition involved subcloning of the wild-type and mutated L chain structural genes into plasmid

expression vectors. The vector employed for this purpose was designed to express a fusion protein that links a maltose protein domain at the N terminus, with L chain sequences at the C terminus.

Our work with BoNT/A began with the subcloning of the L chain protein coding sequence. A DNA fragment encoding the BoNT/A L chain was PCR-amplified using sense and antisense primers that annealed to the 5' and 3' ends of the L chain gene. The amplification product was ligated into the pBluescript II SK$^+$ vector to create plasmid pSAL. Double-stranded plasmid sequencing verified that the nucleotide sequence of the cloned L chain gene was identical to that of the authentic BoNT/A L chain gene.

Example 1 describes the methods used to clone the polynucleotide sequence encoding the BoNT/A L chain.

Example 1

Subcloning the BoNT/A L Chain Gene

The DNA sequence encoding the BoNT/A L chain was amplified by a PCR protocol that employed synthetic oligonucleotides having the sequences, 5'-AAAGGCCTTTTGTTAATAACAA-3' (SEQ ID NO: 1) and 5'-GGAATTCAATGAATAACATAGGAAATAG-3' (SEQ ID NO: 2). Use of these primers allowed the introduction of Stu I and EcoR I restriction sites into the 5' and 3' ends of the BoNT/A L chain gene fragment. These restriction sites were subsequently used to facilitate unidirectional subcloning of the amplification products. Additionally, these primers introduced a stop codon at the C-terminus of the L chain sequence. Chromosomal DNA from *C. botulinum* (strain 63 A) served as a template in the amplification reaction.

The PCR amplification was performed in a total volume of 100 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 50 pmol of each primer, 200 ng of genomic DNA and 2.5 units of *Taq*-polymerase (Promega). The reaction mixture was subjected to 35 cycles of denaturation (1 minute at 94°C), annealing (2 minutes at 37°C) and polymerization (2 minutes at 72°C). Finally, the reaction was extended for an additional 5 minutes at 72°C.

The PCR amplification product was digested with Stu I and EcoR I, purified by agarose gel electrophoresis, and ligated into Sma I and EcoR I digested pBluescript II

SK+ to yield the plasmid called pSAL (Figure 1). Bacterial transformants that harbored this plasmid were isolated according to standard protocols. The cloned L chain gene sequence was verified by DNA sequencing using SEQUENASE (United States Biochemicals) according to the manufacturer's instructions.

To achieve a high level of protein expression, the cloned BoNT/A protein coding sequence was cleaved from plasmid pSAL and ligated into the pMAL-c2 expression vector to form plasmid pCAL. The L chain protein coding sequence in this construct was under transcriptional control of the P_{tac} promoter that can be induced with isopropyl-ß-thiogalactopyranosid (IPTG).

Example 2 describes the methods used to construct a plasmid that directed expression of the BoNT/A L chain in a bacterial host cell.

Example 2

Construction of a Recombinant BoNT/A L Chain Expression Vector

The L chain gene was cleaved from plasmid pSAL by digestion with BamHI and Sal I and then ligated between the BamHI and Sal I sites of the pMAL-c2 expression vector (New England BioLabs). The latter plasmid harbors the *MalE* gene encoding maltose binding protein (MBP) that is controlled by a strong inducible promoter, P_{tac}. A multiple cloning site (MCS) within this plasmid permits subcloning of the L chain gene at the 3' end of *MalE*. Importantly, a cleavage sequence specific for Factor X_a protease is present between *MalE* and the fused L chain gene.

The structure of this clone was verified by restriction enzyme digestion and agarose gel electrophoresis. DNA sequence analysis confirmed its correct orientation when compared to the authentic L chain gene sequence. Sequence analysis also revealed the 5' end of the L chain gene was fused to the MCS and Factor X_a cleavage site via a short sequence that encoded seven amino acids. Moreover, the position of this site showed the L chain gene to be in the same translational reading frame as the *MalE* gene.

With the availability of the pCAL expression plasmid, it was possible to test bacterial clones for expression of the BoNT/A L chain fusion protein.

Example 3 describes the methods used to produce the BoNT/A L Chain Fusion Protein.

Example 3

Expression of the BoNT/A L Chain Fusion Protein

A single fresh bacterial colony containing expression construction was inoculated into L-broth that was made 100 μg/ml ampicillin, and grown overnight at 37°C. The culture was then diluted 1:10 in fresh L-broth containing ampicillin at 100 μg/ml. After a 1 hour incubation, expression of the fusion protein was induced by the addition of IPTG to a final concentration of 0.3 mM. After an additional 4 hour incubation at 37°C, bacteria were collected by centrifugation at 4000 x g for 10 minutes.

Initially, small (10 ml) cultures of *E. coli* that harbored plasmid pCAL were induced with IPTG, and aliquots of solubilized cells were analyzed by SDS-PAGE. This analysis revealed the presence of an induced protein band of roughly 90 kDa M_r. This finding was consistent with the expected size of a fusion protein composed of MBP (40 kDa) and BoNT/A L chain (50 kDa). Such expression in extracts of induced cells was also confirmed by Western blotting with a polyclonal anti-L chain antibody.

Accordingly, total *E. coli* extract or purified proteins were solubilized in SDS sample buffer and subjected to polyacrylamide gel electrophoresis according to the procedure described by Laemmli in *Nature* 227:680 (1970). When required, proteins were transferred from slab gels to PVDF membranes. Western blotting was performed using a rabbit polyclonal anti-L chain antibody (1:500 dilution), and an alkaline phosphatase detection system as described by di Bello et al., in *Eur. J. Biochem.* 219:161 (1993).

In addition to a 90 kDa fusion protein, this antibody also identified a number of lower molecular weight protein bands. This result suggested the presence of extensive enzymatic degradation in those cells. Despite the inability of various protease inhibitors (1 mM phenylmethylsulphonyl fluoride and 10 mM benzamidine) to eliminate this breakdown, the final yield of intact fusion protein remained sufficient. Indeed, on a preparative scale, cells cultured overnight and diluted with fresh medium by 1:10 before induction with IPTG yielded 30-40 mg of total MPB-L chain fusion protein per liter. This latter quantity was estimated from the intensity of protein staining on SDS-PAGE gels.

To purify the fusion protein from bacterial cells, the cytoplasmic extract from a 1 liter culture was subjected to amylose affinity chromatography. Although a

significant proportion of the isolated material was insoluble, about 5 mg of soluble fusion protein was routinely recovered.

Example 4 describes the methods used to purify the recombinant BoNT/A L chain protein from the bacterial clones.

Example 4

Purification of the Fusion Protein and Recombinant BoNT/A L Chain

Using a modification of the lysozyme and detergent method described by Marston in *DNA Cloning: A Practical Approach* (D.M. Glover ed., IRL Press, Oxford 1987), the bacterial cell pellets were resuspended in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, containing 1 mM phenylmethanesulfonyl fluoride and 10 mM benzamidine, and lysed by lysozyme and deoxycholic acid (10 and 40 mg/l of the original culture, respectively). After the incubation with DNase, Triton X-100 (1% w/v) was added and the mixture was vortexed. The lysate was subsequently centrifuged at 15,000 x g for 15 minutes at 4°C. The supernatant was diluted 1:5 with 10 mM Tris-HCl (pH 7.5), containing 1 mM DTT and applied onto a 5 ml amylose affinity column. Unbound proteins were washed through the column with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl containing 0.5% Tween 20 and 1 mM DTT. The bound MBP-L chain fusion protein was eluted with the same buffer containing 10 mM maltose. Eluted fusion proteins were cleaved at 25°C overnight using Factor X_a protease (Promega, Molecular Biology Grade) in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl and 3 mM $CaCl_2$.

An initial test of the ability of the recombinant L chain to act in a manner similar to native BoNT/A entailed comparing the abilities of the two proteins to enzymically breakdown an analog of synaptosomal-associated protein of M, 25 kDa (SNAP-25). Following a 2 hour incubation at 37°C, SDS-PAGE of the reaction mixtures followed by protein staining revealed that authentic BoNT/A degraded a GST-SNAP-25 fusion protein as indicated by a shift in the M, of the stained substrate protein. Importantly, this assay also revealed that the MBP-L chain fusion protein cleaved the SNAP-25 fusion protein in the same way. This result indicated the recombinant form of L chain, even when linked to MBP, exhibited proteolytic activity toward an artificially elongated SNAP-25 substrate.

Example 5 describes the method used to test the enzymatic activities of the recombinant BoNT/A L chain proteins using a modified SNAP-25 substrate.

Example 5

Evaluation of the Proteolytic Activity of MBP-L Chain Fusion Protein Using a Recombinant SNAP-25 Substrate

Because the BoNT/A cleavage of SNAP-25 occurs at a site near the substrate's C-terminus (residues Gln^{197} and Arg^{198}), proteolysis results in a relatively minor decrease in the size of the substrate. Consequently, the mobility of the substrate and the reaction product are not easily distinguished by SDS-PAGE. To circumvent this problem, a recombinant form of SNAP-25 that contained 18 additional amino acids at its C-terminus was used to exaggerate the change in size of the fragment that remained following proteolysis. Use of this modified substrate allowed evaluation of the enzymic activity by SDS PAGE and protein staining. A glutathione-S-transferase (GST) SNAP-25 fusion protein was incubated with native BoNT/A or the recombinant MBP-L chain fusion protein in 50 mM Tris-HCl, pH 8.0 at 27°C for 2 hours. GST was used to provide a quick means of separating the candidate protein from the rest of the cellular components. The reaction was stopped by the addition of SDS sample buffer and boiling, prior to subjecting the samples to SDS-PAGE.

As a further means of testing the biological activity of the BoNT/A recombinant L chain, the ability of the fusion protein to diminish Ca^{2+}-evoked catecholamine release from digitonin-permeabilized bovine adrenochromaffin cells was tested. Consistently, recombinant L chain fusion protein, either intact or cleaved with Factor X_a to produce the free MBP and recombinant L chain, induced a dose-dependent inhibition of Ca^{2+}-stimulated release equivalent to that by native BoNT A.

Example 6 describes the methods used to assess the ability of the BoNT/A fusion protein or free light chain to inhibit catecholamine release from chromaffin cells.

Example 6

Assessing the Ability of Recombinant Fusion Protein or L Chain to Inhibit Catecholamine Release From Permeabilized Chromaffin Cells

Chromaffin cells were prepared from bovine adrenal glands by protease perfusion using the method described by Livett in *Physiol. Rev.* 64:1103 (1984). The cells were plated at 1 x 10^6 cells/well in 24-well plates in Dulbecco's modified Eagle's

medium supplemented with 10% fetal calf serum, 8 μM fluorodoxyurine, 50 μg/ml gentamycin, 10 μM cytosine arabinofuranoside, 2.5 μg/ml fungizone, 25 international units/ml penicillin, 25 μg/ml streptomycin and 2 mM glutamine. Experiments were performed 3-8 days after plating. Ca^{2+}-evoked catecholamine release was measured fluorometrically. Results from this experiment indicated the BoNT/A fusion protein or L chain inhibited catecholamine release from permeabilized cells (Figure 2).

To assess the activity of the recombinant BoNT/A L chain - once cleaved from the MBP fusion protein with Factor X_a - at mammalian motor nerve endings and *in vivo*, it was first reconstituted with H chain isolated from native BoNT/A. Following removal of DTT and urea by dialysis, the association of the recombinant L chain and native H chain to form a disulphide-linked protein roughly 150 kDa in size was monitored by SDS-PAGE and quantified by densitometric scanning. Results from this procedure indicated the proportion of dichain protein formed with the recombinant L chain - when in the heterogeneous fusion protein mixture - was much lower than that formed with the native L chain. Indeed, only about 8% of the recombinant L chain was reconstituted whereas at least 90% of the native L chain reassociated with the H chain.

Example 7 describes the methods used to reconstitute dichain toxins using recombinant L chain and native H chain proteins.

Example 7

Reconstitution of Recombinant L Chain with Purified H Chain

The native constitutive chains of BoNT/A were isolated and purified using established chromatographic procedures following denaturation with 2 M urea and reduction with 100 mM DTT. These procedures have been described by Kozaki et al., in *Japan J. Med. Sci. Biol.* 34:61 (1981), and by Maisey et al., in *Eur. J. Biochem.* 177:683 (1988). Denatured and reduced purified H chain protein was added to an equimolar amount of either native L chain or a Factor X_a cleaved fusion protein mixture containing MBP and recombinant L chain. Reconstitution was carried out by dialysis of the samples at 4°C against 25 mM Tris (pH 8.0), 150 mM NaCl over 4 days. The formation of a 150 kDa M_r covalently-linked heterodimer was assessed by SDS-PAGE under non-reducing conditions. It should be noted that before the MBP and L chain mixture was added to the H chain, any remaining Factor X_a activity was blocked by treatment with 40 μg/ml of soybean trypsin inhibitor for 30 minutes at 20°C.

In order to assess the ability of the recombinant protein to act at the toxin's clinically-relevant target, the reconstituted material was applied to excised mouse phrenic nerve-hemidiaphragms.

Example 8 describes the methods used to test the *in vivo* biological activities of the reconstituted toxins.

Example 8

Assessment of the Mouse Lethality of Reconstituted Toxins and Their Effect on Neuromuscular Transmission

Mouse phrenic nerve-hemidiaphragms were excised from Balb/C mice (20-25 g) and bathed in a closed circulatory perfusion system containing 10 ml of aerated Krebs-Ringer composed of 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 23.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11.7 mM glucose. The solution was adjusted to a pH of 7.4. Muscle twitch was evoked by supramaximal stimulation of the phrenic nerve and measured using a force-displacement transducer as described by Simpson in *J. Pharmacol. Exp. Ther.* 212:16 (1980).

When added to the medium bathing this tissue, the toxin that had been reconstituted using the cleaved fusion protein mixture effectively blocked neuromuscular transmission (Figure 3). In accordance with the aforementioned observations made with adrenochromaffin cells, the potency of the cleaved fusion protein reconstituted toxin was indistinguishable from that of the reconstituted native chains. Importantly, the blockade of neuromuscular transmission by these reconstituted proteins was reversed upon the application of 0.3 mM 4-aminopyridine, a blocker of voltage-gated K^+ channels which is known to temporarily restore nerve-evoked muscle tension at BoNT/A-poisoned synapses. This confirms that the inhibition by the recombinant L chain-containing samples results from a presynaptic blockade of transmitter release and thus mimics that caused by BoNT/A.

Our results to this point indicated that reconstituted recombinant BoNT/A toxin that incorporated the recombinant L chain was functionally similar to the native toxin. It remained to be demonstrated that the reconstituted recombinant BoNT/A toxin functioned *in vivo*.

Example 9 describes the method used to test the activity of the recombinant BoNT/A L chain in laboratory mice.

Example 9

In Vivo Activity of the Reconstituted Recombinant BoNT/A

The ability for the reconstituted samples to induce botulism in laboratory mice was evaluated following their intraperitoneal injection into mice and expressed as the number of doses, lethal within 4 days present per mg of protein (LD_{60}/mg).

Notably, similar results to those detected with the *in vitro* test were obtained when the reconstituted samples were injected into mice; the toxicity of the reconstituted cleaved fusion protein (6×10^7 LD_{50}/mg) was comparable to that of the reconstituted native chains (7×10^7 LD_{50}/mg). Therefore, by all *in vitro* and *in vivo* assays used in the present study, the recombinant L chain expressed in *E. coli* matches the activity of its native counterpart, though the extent of the reconstitution achieved with the recombinant is inferior.

Thus, we have demonstrated that dichain BoNT/A toxin reconstituted from native H chain and recombinant L chain retained many of the properties of the wild-type toxin. We next investigated the effects of mutagenized L chain on proteolytic activity associated with BoNT/A.

To create attenuated L chain proteins that could be tested for functionality, we produced two different mutants of the recombinant BoNT/A L chain gene. These recombinants were produced by mutagenesis of the nucleotide sequences that encoded either of two amino acid positions (Glu^{224} to Gln^{224} and His^{227} to Tyr^{227}). We sought to test whether these mutations in the encoded proteins would, at the same time, compromise L chain enzymatic activity and allow reconstitution with native H chain.

Example 10 describes the methods used to construct polynucleotide sequences encoding BoNT/A L chain proteins that harbor site-directed mutations.

Example 10

Preparation and Expression of BoNT/A L Chain Mutants

To generate mutants using a PCR protocol, the following sense and antisense oligonucleotide primers were synthesized and used as primers during primary amplification together with the two oligonucleotides described in Example 1 to amplify the L chain coding sequence from genomic DNA. The sense and antisense oligonucleotide primers used to create the Gln^{224} mutant respectively had the sequences, 5'-GCACATCAACTTATACAT-3' (SEQ ID NO: 3) and 5'-

ATGTATAAGTTAATGTGC-3' (SEQ ID NO: 4). The sense and antisense oligonucleotide primers used to create the Tyr^{227} mutant respectively had the sequences, 5'-AACTTATATATGCTGGAC-3' (SEQ ID NO: 5) and 5'-GTCCAGCATATATAAGTT-3'(SEQ ID NO: 6).

Secondary PCR under standard conditions, using the two primers previously employed to amplify the wild-type BoNT/A L chain coding sequence, resulted in amplification of the complete mutant genes. As for the wild-type L chain, these amplified products were purified by agarose gel electrophoresis and cloned into the expression vector pGEX-2T which houses the gene encoding GST. Cultures of *E. coli* containing the expression constructs were grown and expression of the fusion proteins induced exactly as for the MBP wild-type L chain fusion protein. Following lysis of the cells and extraction of the cytosol as described before, the GST fusion proteins were purified by glutathione affinity chromatography.

Given the availability of the mutant L chain proteins, it was of interest to evaluate the effect of mutation on proteolytic activity.

Example 11 describes the methods used to assess the proteolytic activity of the mutant BoNT/A L chain proteins.

Example 11

Characterization of the Mutant L Chain

Once isolated, the proteolytic activity of GST mutated L chain Gln^{224} and Tyr^{227} fusion proteins toward a recombinant form of the substrate for BoNT/A, SNAP-25, was assessed. After incubation of the purified mutant L chains and the GST-SNAP-25 substrate in 50 mM Tris-HCl (pH 8.0) for 2 hours or at 22°C overnight, the products were analyzed by SDS-PAGE. It was found that neither of the two mutants displayed any detectable proteolytic activity toward the SNAP-25 analog. In contrast, the wild-type recombinant L chain fusion protein exhibited strong proteolytic activity as evidenced by the expected cleavage pattern on the stained protein gels.

These results proved that alteration of a single amino acid position in the BoNT/A L chain protein sequence eliminated proteolytic activity of the resulting fusion protein.

The foregoing procedures that relate to mutant BoNT/A L chain proteins were performed using GST-based fusion proteins. Those of ordinary skill in the art will

appreciate the high likelihood that other L chain fusion proteins will similarly lack proteolytic activity.

Example 12 describes alternative methods that can be used to generate mutant L chain MBP fusion proteins.

Example 12

Expression of Mutant MBP BoNT/A L Chains

Expression plasmids that harbor either the Gln^{224} or the Tyr^{227} mutant L Chain coding sequences are constructed as described above except that the mutant sequences that were generated according to the PCR mutagenesis protocol are substituted for the wild-type sequences. Accordingly, mutant L chain gene sequences are fused with the maltose binding protein (MBP) gene (mal E) in the plasmid, pMAL-c2. After induction with IPTG, the MBP-L chain fusion proteins reach levels of production that approximate those observed when the wild-type L chain sequences were employed, as discussed above. The mutant fusion proteins are then purified according to standard protocols. Neither the L chain MBP fusion proteins, nor the factor X_a cleavage products exhibit proteolytic activity against a recombinant GST SNAP-25 fusion protein substrate.

Furthermore, when reassociated with native BoNT/A H chain protein, the resulting mutant dichains have none of the toxic activities ordinarily associated with the wild-type toxin. However, the inactive dichain competes with wild-type BoNT/A for receptor-binding. This result indicates the mutant dichain interacts with the cognate toxin receptor.

These findings are taken to indicate the inactive dichain is an excellent candidate as a transporter for the delivery of linked drug molecules to neuronal target cells.

The modified BoNT/A toxin transporters described above will have numerous clinical applications. For example, the BoNT/A-based transporters can be use to deliver therapeutically useful drugs to the peripheral motor terminal. Accordingly, these drugs delivered in this fashion will be useful in controlling limited numbers of muscle groups. Among the maladies that will be investigated as therapeutic targets are: tardive dyskinesia, spastic colitis, essential tremor, smooth muscle abnormalities, localized spasticity, painful muscle spasms localized to back or other muscle groups, temporal mandibular disorders, spasmodic dysphonia and tension headaches.

Example 13 describes how the native or recombinant botulinum toxin L chain proteins can be covalently linked to a chemical compound. In this Example, a drug that blocks uptake of acetylcholine from the cytoplasm to the synaptic vesicle is linked to the transporter protein using free SH groups. The synthetic pathway employed in this procedure is diagrammed in Figure 4.

Example 13

Chemical Bonding of Transporter Protein and Vesamicol

Vesamicol is first attached onto a linker of 1-chloropropyl 12-chloro-dodecanoate, using equimolar concentrations of each in a base catalyst solution (such as pyridine, 2,6-dimethylpyridine, triethylamine or tetramethylguanidine) in solvents such as THF, DMSO, DMF or acetonitrile (Figure 4). The reaction is performed at temperatures of between 0 and 100°C for from 1 to 48 hours. The resulting vesamicol-linker product is then reacted with equimolar amounts of the potassium salt of maleimide in the same solvents, as above, and in the presence of sodium iodide (used as a catalyst) using similar times and temperatures as above.

The recombinant inactive L chain and native H chain subunits are renatured to produce a dichain molecule of roughly 150 kDa M_r. Renaturation is accomplished by mixing equimolar amounts of L chain and H chain proteins in the presence of urea and dithiothreotol. The mixture is dialyzed at 4°C against a Tris-NaCl, (pH 8.0) buffer that contains, in mM, NaCl, 460; KCl, 10; $CaCl_2$, 11; $MgCl_2$, 25; $MgSO_4$, 28; Tris-HCl, 10. The buffer is preferably oxygenated during the renaturation process. The buffer is changed 5 times over 24 hours. The removal of urea and DTT leads to the disulfide linkage of the L chain and H chain. Each dichain has several free sulfhydryl groups that are available for drug attachment.

The vesamicol linker is bonded to the free sulfhydryl groups found on the intact transporter molecule by mixing a 5 fold molar excess of the vesamicol linker with the transporter in Tris-NaCl, described above, at 4°C in the dark for 1 to 24 hours.The transporter-vesamicol preparation is then dialyzed against Tris-NaCl overnight to remove excess vesamicol-linker-maleimide from the vesamicol transporter.

The drug-botulinum toxin material is then available for administration as a sterile injection in a therapeutically effective dose. The modified and inactivated botulinum neurotoxin transporters described above will have numerous clinical applications. Example 14 describes how the chemically modified inactive BoNT/A toxin transporter

described above can be used as a therapeutic agent for delivering chemical compounds to neurons that express toxin receptors.

Example 14

Therapeutic Administration of Modified Toxins:

Tardive Dyskinesia

A male patient, age 45, suffering from tardive dyskinesia resulting from the treatment with an antipsychotic drug, such as Thorazine or Haldo, is treated with therapeutically effective doses of an appropriate drug, as would be appreciated by one of ordinary skill in the art, attached to an inactive botulinum toxin transporter directly into the facial muscle muscles. After 1-3 days, the symptoms of tardive dyskinesia, i.e., orofacial dyskinesia, athetosis, dystonia, chorea, tics and facial grimacing, etc. are markedly reduced.

Example 15 further illustrates how the chemically modified inactive toxins described above can be used as therapeutic agents for delivering chemical compounds to neurons that express toxin receptors.

Example 15

Therapeutic Administration of Modified Toxins:

Essential Tremor

A male, age 45, suffering from essential tremor, which is manifested as a rhythmical oscillation of head or hand muscles and is provoked by maintenance of posture or movement, is treated by injection with therapeutically effective doses of a drug (see list in previous table in patent application) attached to an inactive botulinum toxin transporter directly into the affected muscles. The muscles may be identified with the aide of electromyography (EMG). After one to two weeks, the symptoms are substantially alleviated; i.e., the patent's head or hand cases to oscillate.

Example 16 further illustrates how the chemically modified inactive BoNT/A toxin transporter described above can be used as therapeutic agents for delivering chemical compounds to neurons that express toxin receptors.

Example 16

Therapeutic Administration of Modified Toxins:

Smooth Muscle Abnormality

A female, age 30, with a constricted lower esophagus (disease called Achalasia) manifests symptoms which prevent food ingestion. Due to the lower esophagus

contraction, food and fluid accumulate and eventually is regurgitated, preventing the patient from obtaining adequate nutrition. Therapeutically effective doses of a drug (see list in previous table in patent application) attached to an inactive botulinum toxin transporter is administered directly into the affected sphincter muscles. Usually the injections are administered in 2 to 4 quadrants with any endoscopic device or during surgery. In about 1-7 days, normal passage of solids and liquids into the stomach is achieved resulting in an elimination or reduction in regurgitation.

Example 17 further illustrates how the chemically modified inactive BoNT/A toxin transporter described above can be used as a therapeutic agent for delivering chemical compounds to neurons that express toxin receptors.

Example 17

Therapeutic Administration of Modified Toxins:
Spasmodic Dystonia (Overactive Vocal Chords)

A male, age 45, unable to speak clearly, due to spasm of the vocal chords, is treated by injection of the vocal chords by injection of therapeutically effective doses of an appropriate drug, as would be appreciated by one of ordinary skill in the art, attached to an inactive botulinum toxin transporter. After 1 to 7 days, the patient is able to speak clearly.

In summary, we have gained further insight into the action of the BoNT/A toxins by employing recombinant DNA techniques to produce L chain protein in useful quantities. Utilizing a PCR-based protocol, the gene encoding the L chains were amplified, subsequently cloned into expression vectors and expressed at a high level in *E. coli.* After purification from the cytosolic fraction using amylose affinity chromatography, the fusion protein was found to proteolytically cleave a recombinant form of the substrate for BoNT/A, synaptosomal-associated protein of M, 25 kDa (SNAP-25). Moreover, once enzymatically cleaved from the maltose binding protein, the recombinant L chain proteins were shown to exhibit properties like those of the native proteins. Also, the expressed L chains were reconstituted with purified native H chains to form disulphide linked, dichain proteins which inhibited nerve-evoked neuromuscular transmission *in vitro* and produced the symptoms of botulism in mice.

Most significantly, we also discovered that single amino acid substitutions in the sequence of the L chain proteins completely abrogated the proteolytic activity ordinarily

associated with the wild-type proteins. This now allows the formation of dichain toxins that are attenuated by virtue of incorporating a proteolytically inactive L chain subunit .

We also anticipate that single genes that incorporate appropriate site directed mutations can be produced for each of the neurotoxins so that attenuated toxins can be produced in bacteria. This approach will advantageously avoid the need to reconstitute dichain molecules from subunit components. The resulting attenuated toxin can advantageously serve as a transporter for delivering covalently linked chemical compounds to neuronal cells that express toxin receptors.

Abstract of the Invention

A chemical conjugate for treating a nerve cell related disorder is provided. This conjugate includes an active or inactive botulinum toxin having specificity for a target nerve cell. The toxin is conjugated to a drug or other bioactive molecule without affecting the toxin's ability to enter the target nerve cell.

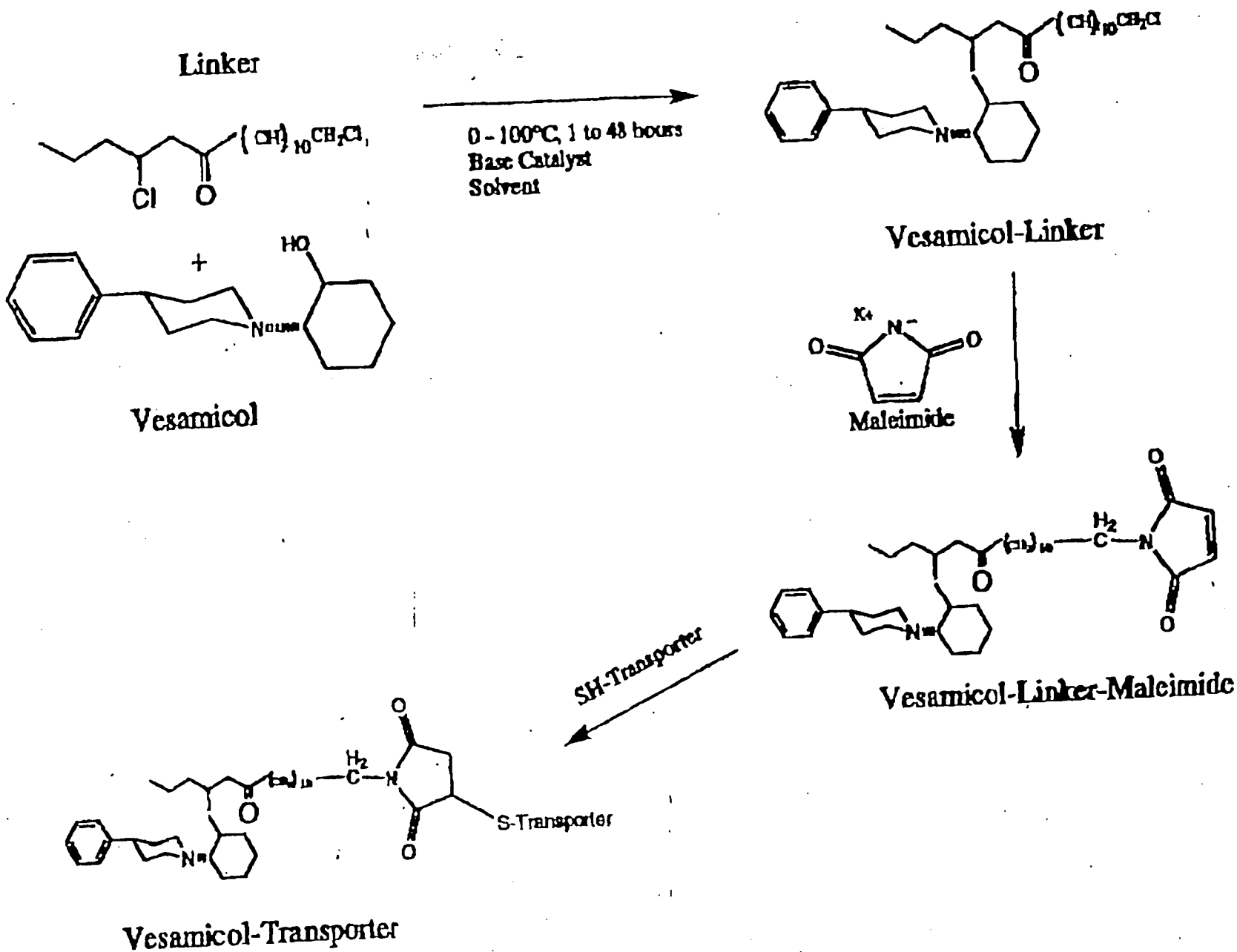

Linker
Cl
O
0 - 100°C, 1 to 48 hours
Base Catalyst
Solvent
HO
+
Vesamicol
Vesamicol-Linker
Maleimide
Vesamicol-Linker-Maleimide
SH-Transporter
S-Transporter
Vesamicol-Transporter

Fig. 4